PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Remacle et al.
Appl. No.	:	10/606,162
Filed	:	June 25, 2003
For	:	METHOD FOR THE DETERMINATION OF CELL ACTIVATION
Examiner	:	Petersen, Clark D.
Group Art Unit	:	1657

DECLARATION UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate the phosphorylation of CDK8 protein.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

4. Claim 15 is drawn to a method for evaluating cell activation by evaluating the phosphorylation state of specific proteins from the list of Table 1 in the specification. The claim is now restricted according to restriction election filed May 2, 2006 to the determination of cell activation by measuring the phosphorylation of CDK8, PAK6 and MKK3. There was no evidence in the literature for the phosphorylation of CDK8. We thus conducted experiments to assess the phosphorylation of CDK8 protein.

5. Exhibit 1 attached hereto shows signal of phosphorylation of CDK8. The example describes the phosphorylation of CDK8 by protein kinase A and reagents used for detecting this phosphorylation. As illustrated in the accompanying Exhibit, CDK8 is phosphorylated on two sites by protein kinase A.

6. In the literature, it is not clear what mechanisms regulate CDK8 function. We provide here evidence that phosphorylation of CDK8 is possible and is an effective means for determining cellular activation state. Therefore, Claims 1 and 15 which are drawn to the elected species of the combination of CDK8, PAK6 and MKK3 are enabled.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 5 June 2007 By: _____
Jose Remacle

Exhibit 1: Signals of phosphorylation of CDK8 protein.

To establish that CDK8 is phosphorylable, we first checked the protein sequence for the presence of kinase phosphorylation sites. The human CDK8 sequence was used. CDK8 is a potential substrate for protein kinase A, as it contains four putative PKA phosphorylation sites on Thr31, Ser114, Thr287 and Thr325 (human protein numbering). The kinase accessibility to these four sites is unknown, as the CDK8 structure is not solved to date.

As a second step, the in vitro phosphorylation of CDK8 by protein kinase A was performed with radioactive ATP as follows:

250 ng CDK8 (recombinant human protein fused to a 6x His-tag; Mr = 59 kDa; Autogen Bioclear, UK) were incubated with 2500 units of the PKA catalytic subunit (New England Biolabs, USA) in 1x PKA phosphorylation buffer (New England Biolabs, USA) in the presence of 200 mM ATP (New Englan Biolabs, USA) and 100 µCi [γ 32P] ATP (Amersham Biosciences, USA) in a 30 µl final volume. Reaction was performed for 1 hour at 30°C. A control reaction was performed in the absence of the PKA catalytic subunit.

Ten µl of these reactions (test and control) were analyzed by electrophoresis under denaturing conditions: reactions were supplemented with 4 µl sample buffer and 2 µl EDTA (InVitrogen, USA) and run at 200 V on a NuPAGE MOPS gel (NuPAGE system, InVitrogen, USA). The SeeBlue Plus2 prestained standard (InVitrogen, USA) was loaded onto the gel to check for the molecular mass of the radioactive bands. Results were analyzed by phosphor imaging using the Cyclone phosphor imager (Perkin Elmer, USA).

Figure 1 shows two radioactive bands in the test reaction, with molecular masses being higher than the 59 kDa of the native protein. The increase of Mr can be explained by the presence of one or two phosphate residues which interfere with the SDS binding and gives an apparent increase of the Mr of approximately 5 kDa. This result demonstrates the phosphorylation of CDK8 by PKA.

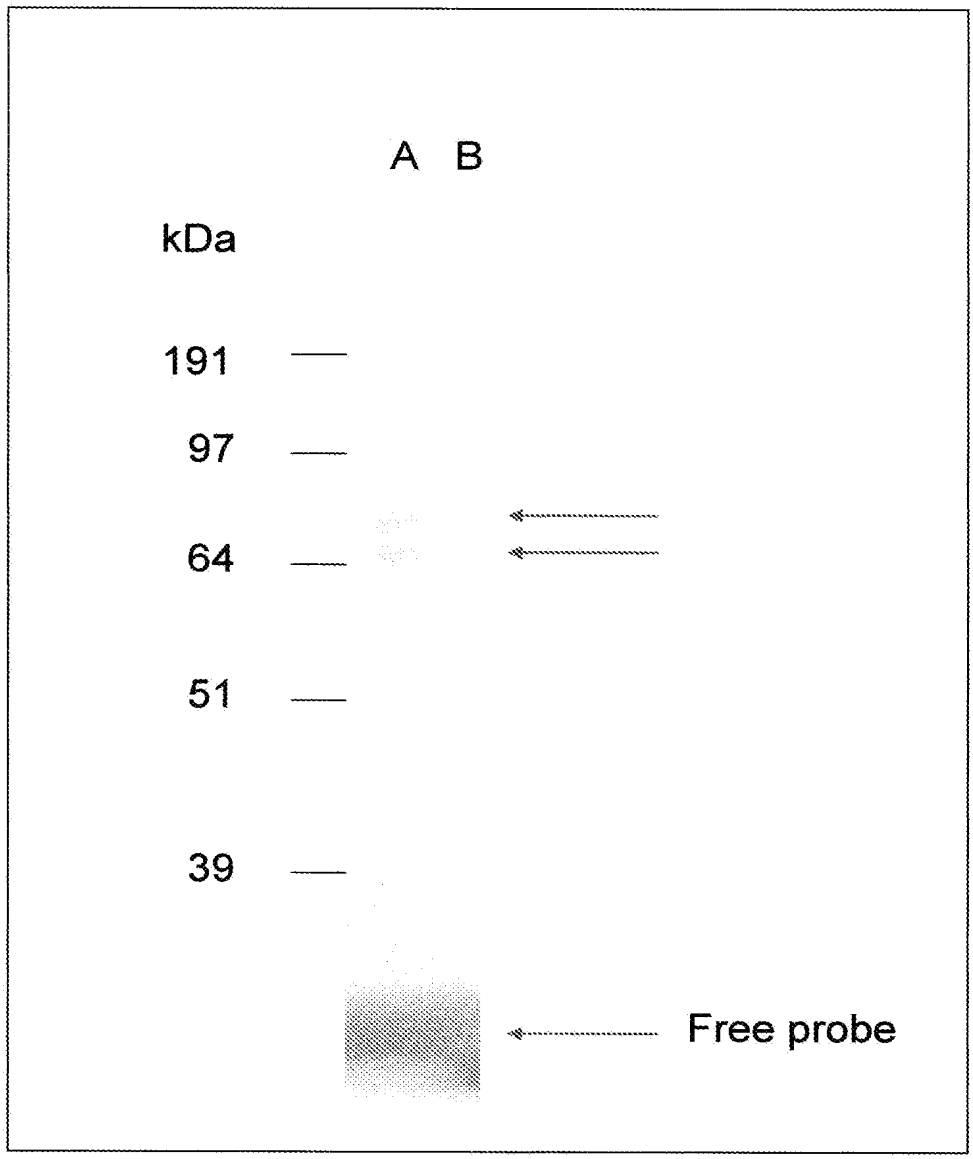

Figure 1: recombinant human CDK8 (His-tagged) was subjected to an in vitro phosphorylation in the presence (A) or absence (B) of protein kinase A using [γ 32P] ATP. The protein phosphorylation was assessed after electrophoresis by phosphorimaging.
The red arrows point to two radioactive CDK8 forms of respectively 64 and 69 kDa.
The free probe is visible at the bottom of the gel.